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SCANNING ELECTRON MICROSCOPY OF THE REGENERATED SHELL OF THE MARINE ARCHAEOGASTROPOD, *TEGULA*

CHARLENE REED-MILLER

Department of Geology, Florida State University, Tallahassee, FL 32306

ABSTRACT

A window was cut in the first body whorl of the marine snail, *Tegula,* to induce shell regeneration. At various intervals after the shell window was cut, the window with the regenerated material and the shell surrounding it were prepared for scanning electron microscopy. Initial crystal deposition occurred in association with an organic matrix and appeared as small, spindle-shaped crystals formed by the aggregation of needle-like subunits. The spindles were frequently aggregated into stellate clusters that coalesced to form a sheet of mineralized tissue. After about two months of regeneration, dumbbell-shaped crystal aggregates and spherulites were apparent on the surface of the regenerated shell. The regenerated shell assumed a normal structure after at least four months of regeneration.

Crystal deposition also occurred on the normal shell bordering the shell window. The crystals assumed several forms, and their orientation appeared to be determined by the microtopography of the underlying shell.

INTRODUCTION

Molluscan shell mineralization is the result of a complex and delicate association of biological, chemical, and physical processes. The result of the interaction of these factors is not always the same, even in a single animal. The degree of organic *versus* inorganic control of mineralization in the molluscan shell is an example of variability in structure determined by the interplay of these three processes. Molluscan growth surfaces show variation in organic and inorganic mechanisms of crystallization. Organic suppression of natural crystal form of the outer (distal) shell layer was much less than in the inner three shell layers of an archaeogastropod, *Cittarium pica* (Wise and Hay, 1968a, b). The same was found to be true for five species of the archaeogastropod genus, *Tegula* (Reed-Miller, 1981a). The aragonitic crystals of the nacreous shell layer are often present in tabular or diminished "c" axis form. This differs from the usual conformation of inorganically precipitated aragonite, elongate twinned prisms, and represents another example of organic control of crystal morphology.

The mineralized product formed during shell regeneration can be similar to, or quite different from the ultrastructure of the normal shell. This emphasizes again structural range of mineralized tissue (Saleuddin and Wilbur, 1969; Wilbur, 1972; Wong and Saleuddin, 1972). Earlier reports showed differences in the structure of regenerated shell compared to the normal shell of *Tegula* (Reed-Miller *et al.,* 1980; Reed-Miller, 1981a). The region of the shell involved in regeneration is considered to be another area of active calcification and mineralization. Since the area of least suppression of natural crystal form occurred at the lip, or growing edge of the shell in some archaeogastropods, including *Tegula* (see above), it was of interest to look at the crystal structure in regenerated *Tegula* shell. The initial ultrastructural changes

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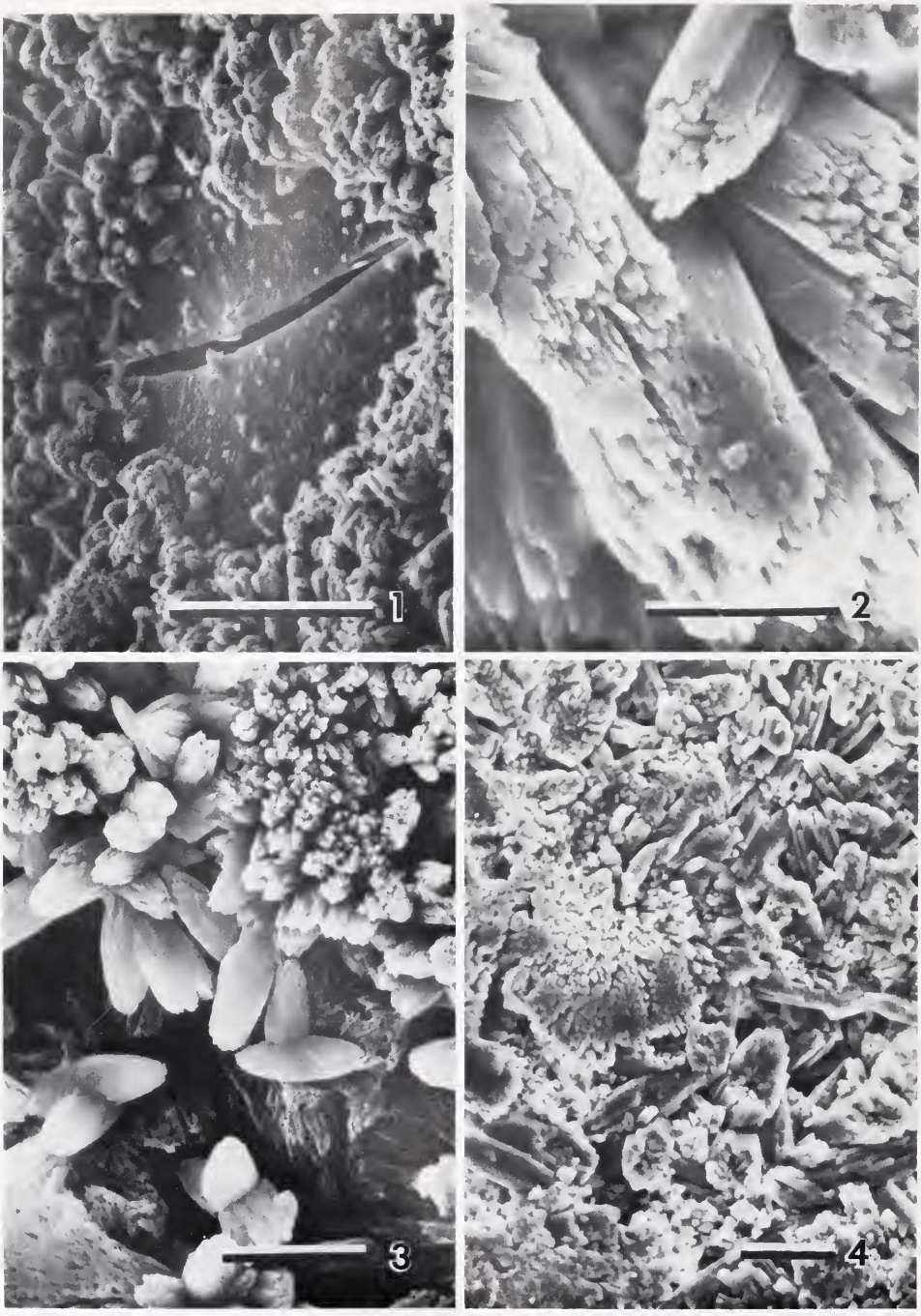

FIGURE 1. Regenerated material in the shell window, showing doubly-pointed crystallites grouped into bundles or rosettes on an organic membrane. One week of regeneration. Bar = 50 μm.

FIGURE 2. Higher magnification of spindle-shaped crystals similar to those shown in Figure 1. Note the elongated, needle-like subunits that make up the spindles. One week of regeneration. Bar = 10 μm.

FIGURE 3. Rosette-shaped assemblages of crystalline spindles. Note the underlying layer of coalesced crystals. Two weeks of regeneration. Bar = 10 μm.

FIGURE 4. A sheet of mineralized tissue formed by the coalescence of rosette-shaped crystal aggregates. Three weeks of regeneration. Bar = 20 μm.

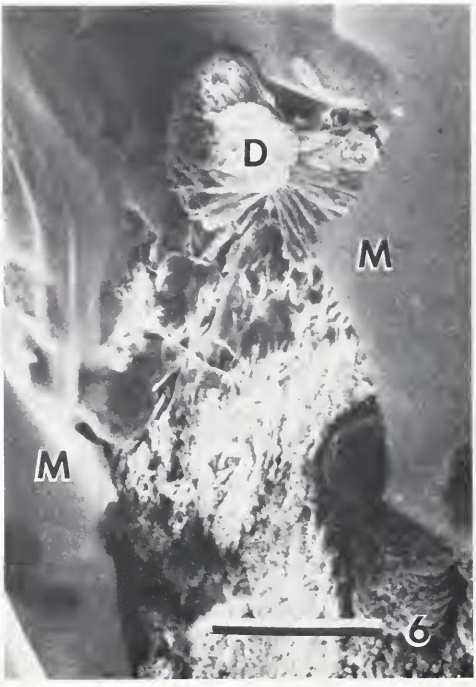

FIGURE 5. Spherulites formed of radiating clusters of needles. Three weeks of regeneration. Bar = 10 μm.

FIGURE 6. Regenerated shell with dumbbell-shaped crystal aggregates (D). Note the organic matrix (M). Two months of regeneration. Bar = 100 μm.

in the mantle, foot, and hepatopancreas during shell regeneration in this marine snail have been reported (Reed-Miller, 1983). The present study was undertaken to describe the ultrastructure of regenerated shell in *Tegula,* and to outline a possible mechanism for the crystal formation.

Preliminary accounts of this work were presented to the American Society of Zoologists (Reed-Miller, 1981b; 1982) and to the American Malacological Union.

MATERIALS AND METHODS

Tegula funebralis and *Tegula eiseni* were obtained from the Pacific Biomarine Laboratories, Inc., Venice, California. They were maintained in aquaria in filtered, aerated sea water from the Gulf of Mexico (32 ppt) at 15°C. The animals were fed marine algae from a laboratory culture.

A 4 mm^2 section of shell was carefully removed from the first body whorl of the shell using a Dremel "Moto-tool," jeweler's saw and a triangular file. Care was taken not to injure the underlying tissue. The opening in the shell, or window, was covered with a small piece of plastic coverslip, and covered with warm dental wax, sealing the window from the external environment.

The regenerated material was removed from the animals (the procedure follows below) at intervals from 6 hours to 6 months after the shell window was cut. These were six, 12, and 18 hours; one, two, three, seven, and ten days; two weeks; and then on a weekly basis up to six months. The experiments were repeated three times with at least four experimental animals examined each time.

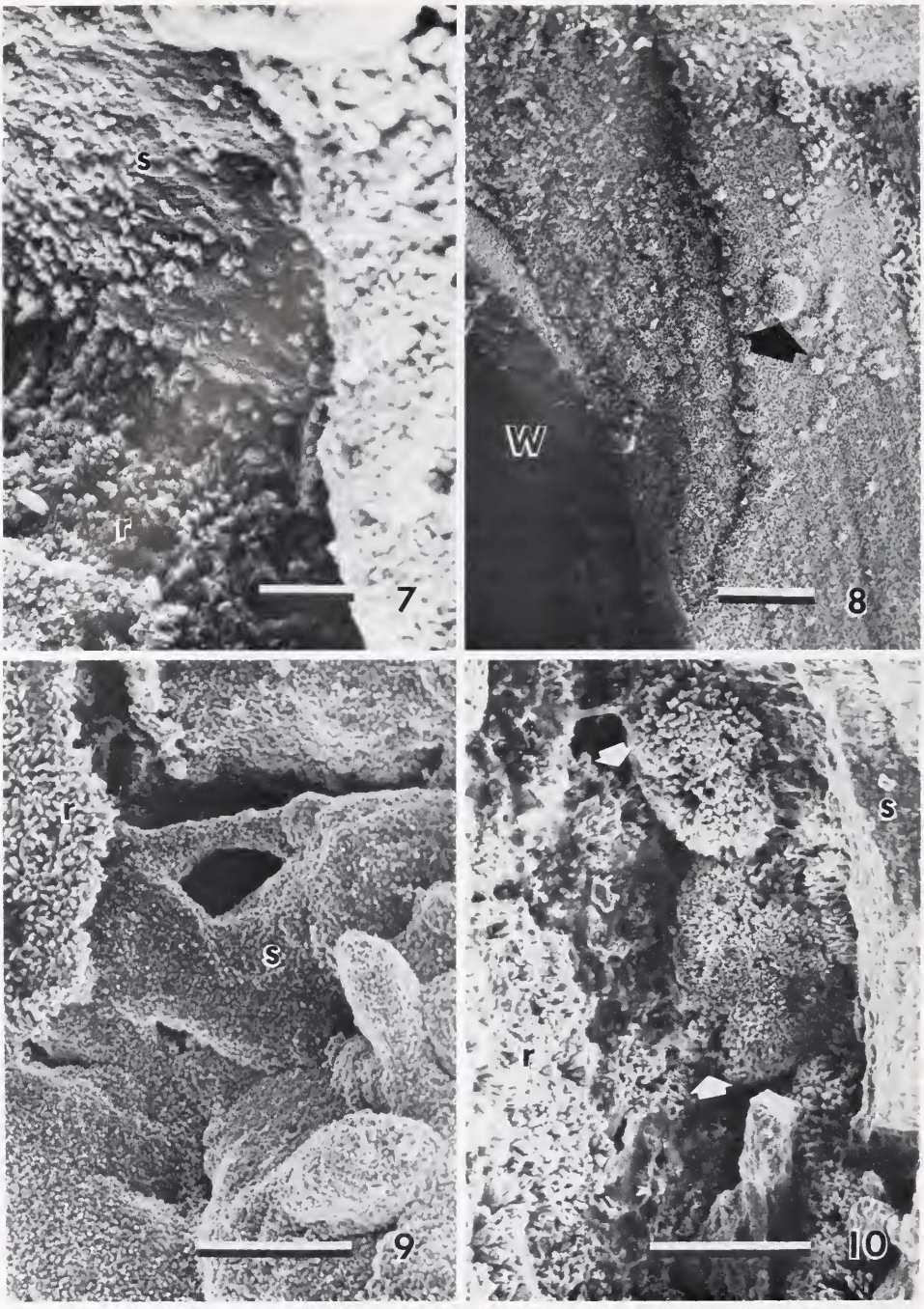

FIGURE 7. The edge of the shell window showing spindle-shaped crystals dotting the normal shell (S) and forming the regenerated shell (R). One month of regeneration. Bar = 10 μm.

FIGURE 8. An area of the shell near the window (W) showing the clustering of spindle-shaped crystals into spherulites. Arrow points to one large spherule. The regenerated shell has been removed. Two months of regeneration. Bar = 100 μm.

FIGURE 9. Inside of the shell at the juxtaposition of regenerated shell (R) and normal shell (S). Note the pavement of small crystals obscuring the normal shell. 2½ months of regeneration. Bar = 100 μm.

FIGURE 10. Edge of the shell window viewed from the inside of the shell with the regenerated material

FIGURE 11. Small polygonal crystallites that were occasionally seen on the normal shell surrounding the shell window. Three months of regeneration. Bar = 4 μm.

FIGURE 12. Elongated trends of needle clusters that were seen on the normal shell bordering the window. Three months of regeneration. Bar = 5 μm.

Scanning electron microscopy

The soft parts were removed from the shell, and the shell was preserved in 70% ethanol, until it was prepared for scanning electron microscopy. The shell was then carefully cut around the window with a rotary rock saw until a small frame of shell (about 3 mm wide) surrounded the window on all sides. This frame and the shell window with the regenerated material were rinsed with distilled water and air dried. The samples were mounted on aluminum scanning electron microscopy stubs with nail polish, and coated with 100–200 Å of gold-palladium (60:40), using an E5100 Polaron Sputter Coater. The material was observed with a Cambridge S4-10 scanning electron microscope operated at 20 kV.

RESULTS

Most of the regenerated shell of *Tegula* was built up from spindle-shaped crystals associated with an organic matrix (Fig. 1). The spindles were made up of smaller, elongated crystallites (Fig. 2). The doubly-pointed crystallites grew and formed radiating

(R) on the left. Note the large radial clusters of crystals emanating from the normal shell (S) on the right. Arrows show some of the contacts between the regenerated shell and growth from the normal shell. Organic matrix is visible overlying some of the regenerated shell in the window. Two months of regeneration. Bar = 100 μm.

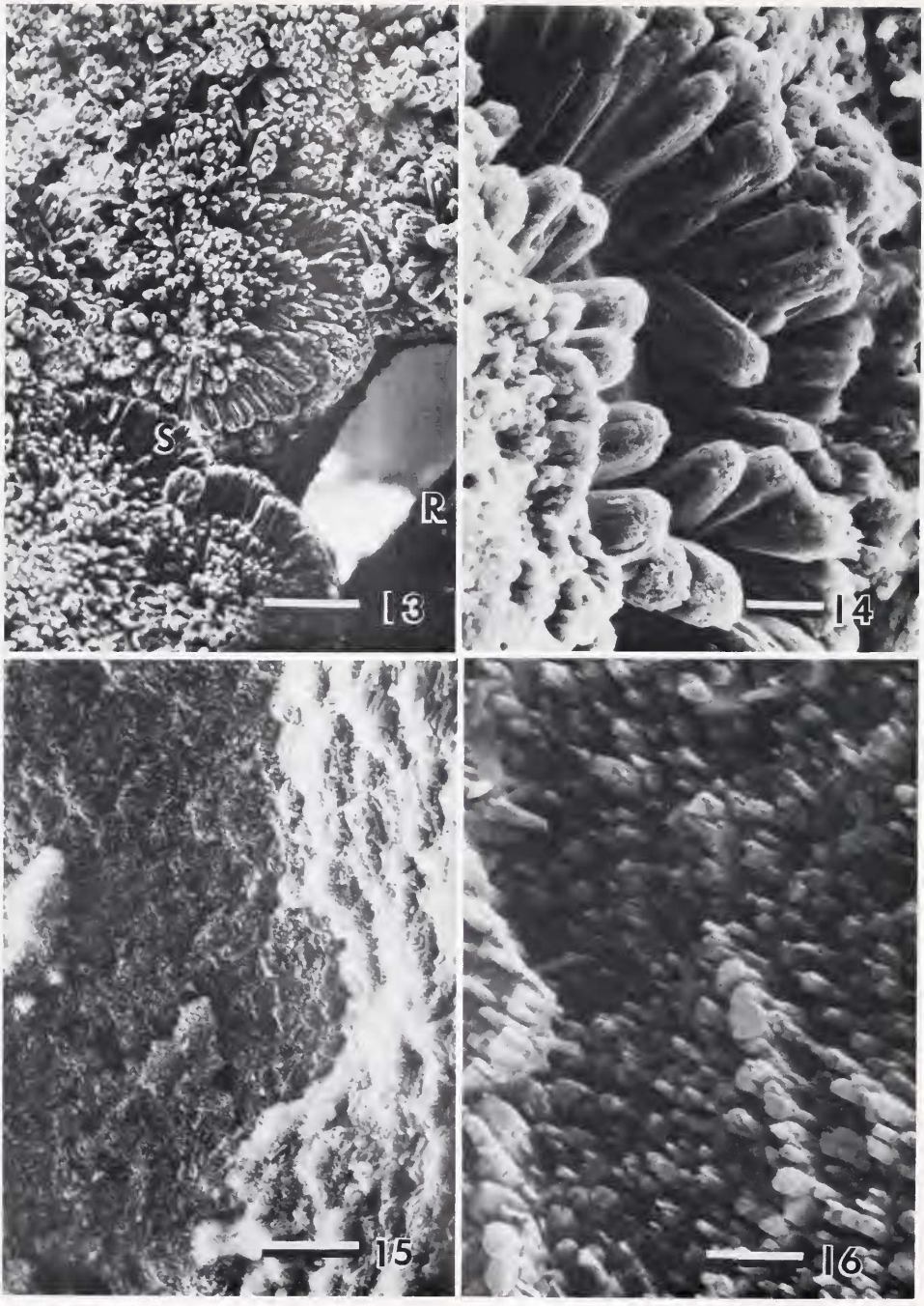

FIGURE 13. The edge of the shell window showing radiating clusters of rod-shaped crystals on the shell (S) surrounding the window. The regenerated shell (R) has separated from the normal shell in this micrograph. Four months of regeneration. Bar = 20 μm.

FIGURE 14. Higher magnification of rod-shaped crystals similar to those shown in Figure 13. The organic matrix has collapsed over the tops of some of the crystals. Four months of regeneration. Bar = 10 μm.

FIGURE 15. An area of shell where the nacreous layer was fractured during the removal of the shell window. Small crystallites pave the surface of the fractured shell, and the hexagonal outlines of the nacre tablets are visible underneath them. The shell window is just out of view at the top of the figure. Three months of regeneration. Bar = 20 μm.

clusters or rosettes (Fig. 3) that eventually coalesced into a mineralized sheet (Fig. 4). Spherules formed of needle-like crystals were also observed (Fig. 5). After about two months of regeneration, dumbbell-shaped crystal aggregates and spherulites associated with an organic matrix were predominant on the surface of the regenerated material (Fig. 6).

Frequently, mineral was deposited on the normal shell surrounding the shell window. This occurred on the edges of the window both inside (next to the mantle) and outside of the shell. Typically the crystals were small and spindle-shaped (Fig. 7), and covered about one to two mm of the normal shell bordering the window (Fig. 8). The area of shell next to the mantle usually showed thicker deposition than the region on the outside of the shell (Fig. 9). After about two months of shell regeneration, crystals of regenerated shell inside the window and crystals growing from the frame around the window made contact in some places (Fig. 10).

The microstructure of the crystallites deposited on the normal shell varied somewhat from the doubly-pointed crystals described for regenerated shell. Polygonal (Fig. 11) and elongated aggregate needles (Fig. 12) were common. After four months of regeneration, the crystals on the edge of the shell window were large and rod-shaped, and were usually assembled in radiating clusters (Fig. 13). The rods were composed of smaller subunits (Fig. 14).

Figure 15 shows crystal deposition over nacreous shell. Small crystallites dot the shell, and outlines of the nacre tablets are discernable. The "c" axes of the crystallites deposited along the edges of the normal nacre tablets are slightly more elongated than those axes of the crystallites deposited on the more central regions of the tablets (Fig. 16). The regenerated shell attained a normal ultrastructural appearance after at least four months of regeneration.

Discussion

There is a striking resemblance between the crystalline structures reported in this paper and structures described in other molluscan shells and for inorganically precipitated aragonite. This similarity has led to a four part hypothesis for the phases of shell regeneration in *Tegula.*

1. Aragonitic needles are precipitated from a carbonate-rich solution onto an organic matrix where they grow and form regenerated shell. The regenerated shell of *Tegula* was built up from aragonitic needle clusters that formed dual tapered crystal spindles. According to this part of the hypothesis, however, regeneration involves precipitation from a solution, and the exact area of deposition may not be limited to the shell window. This was found to be the case for *Tegula.* Crystallites were found on a small region of the nacreous shell bordering the window. The crystallites were typically smaller than the underlying nacre tablets, and in some cases, appeared to conform to or be guided by the pattern imposed by the shape of the individual nacre tablets (See Figs. 15, 16). Schroeder (1973) examined Pleistocene gastropod shells and found that apparently inorganically precipitated aragonite needles lined the interiors of the shells. The needles were oriented in two directions, determined by the underlying crossed lamellar shell structure. Meenakshi *et al.* (1974a) showed that the substrate microtopography influenced calcification patterns during shell regeneration in *Otala lactea,* a land snail. Alexandersson (1974) stated that even during inorganic

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FIGURE 16. Higher magnification of Figure 15 showing elongate "c" axes of the crystallites on the edges of the nacre tablets. Three months of regeneration. Bar = 2 μm.

precipitation, the organic matrices and matrix derivatives have some control over the form of skeletal carbonates.

The crystals described in and around the regenerated shell closely resemble the morphology of inorganically precipitated aragonite crystals (See Ginsburg and Schroeder, 1973 for a description of inorganically precipitated aragonite). In fact, Wind and Wise (1976) noted in their study of spine mineralization in the archaeogastropod *Guildifordia triumphans,* that it was virtually impossible to determine where organically precipitated aragonite ended and inorganically precipitated aragonite began. Note Figures 7, 9, and 10 in this paper which are micrographs of mineralization close to and around the edge of the shell window. It is impossible to discern whether these crystals are of an organic or an inorganic origin. Similar aragonitic crystals have been described filling in and lining gastropod shells in cup shaped algal reefs (Ginsburg *et al.,* 1971; Schroeder, 1972a, b; Ginsburg and Schroeder, 1973), forming the skeletons of one order of green algae (Marszalek, 1971), and as algal cement (Alexandersson, 1974).

2. The needles aggregate to form doubly-pointed bundles, or spindle-shaped crystals associated with an organic matrix. Spindle-shaped crystals have been described in the regenerated shell of other molluscs. For example, Blackwelder and Watabe (1977) and Meenakshi *et al.* (1974b) reported the occurrence of spindle-shaped crystals in the regenerated shells of the freshwater gastropod, *Pomacea paludosa,* and the cephalopod, *Nautilus macromphalus.* In addition, crystals morphologically similar to those described in the regenerated shells have been described in calcified byssi of the bivalve, *Anomia simplex,* and on the surface of the lithodesma of another bivalve, *Lyonsia floridana* (Prezant, 1982).

The random orientation of the crystal spindles in the regenerated shell of *Tegula* parallels the description of the formation of the growth stops and spine diaphragms in *Guildifordia triumphans* (Wind and Wise, 1976). These authors pointed out that the unpatterned disposition of the spindles indicated that they probably began forming in the extrapallial fluid, and settled at random.

3. The spindle-shaped crystals form spherules in one of two ways as outlined by Watabe (1981). First, by additional growth, the spindles become grouped into stellate- or rosette-shaped aggregates that eventually become spherules. Rosettes of spindle-shaped crystals were a prominent component of the regenerated shell in *Tegula.* They coalesced to form a mineralized sheet in the shell window. Spherulitic aggregates of crystals have been observed in other molluscs where shell is being filled in or repaired. Wind and Wise (1976) describe "elongate trends of radiating aragonite needle clusters" filling in the spine cavities of *Guildifordia triumphans,* and Watabe (1981, Fig. 5) showed spherulites of aragonite formed during early shell regeneration in the terrestrial snail, *Cepaea nemoralis.* Moreover, these aggregate crystals have been found in the normal shells of the archaeogastropod, *Cittarium pica* (Wise and Hay, 1968a, b; Erben, 1971).

The second possibility for the mechanism of spherule formation is by the addition of needles to the ends of the spindle-shaped crystals, forming a dumbbell shape. Filling in the midregion of the dumbbell with more needles would result in radial development and spherule formation. After about two months of shell regeneration, large dumbbell-shaped crystals as well as spherules were evident in the regenerated shell of *Tegula.* These crystal structures were also evident in the regenerated shell of *Mytilus edulis,* a marine bivalve, and *Pomacea paludosa,* a freshwater snail (Uozumi and Suzuki, 1979; Blackwelder and Watabe, 1977).

The results of the present study indicate that the stellate- or rosette-shaped clusters of crystal spindles occur during early shell regeneration, and the dumbbell-shaped

aggregates are present during a later stage of regeneration. This is not a definitive statement for all shell regeneration, but examples such as those of *Mytilus* and *Pomacea* show that these crystal types can occur under a wide range of conditions in regenerated shell.

4. Finally, the crystals derived from the rosette-like or the dumbbell-shaped crystal aggregates are closely apposed, and competitional growth results in their coalescence and the formation of a spherulitic prismatic type of shell layer. Micrographs of the regenerated shell after at least three months of regeneration show this type of layer in *Tegula* (Reed-Miller, unpub.). This shell structure has also been seen in the regenerated shell of *Pomacea paludosa* (Blackwelder and Watabe, 1977), the shells of *Cittarium pica* (Wise and Hay, 1968a, b; Erben, 1971), *Nautilus* (Erben *et al.*, 1969; Mutvei, 1972; Meenakshi *et al.*, 1974b), and in bivalve ligaments (Mano and Watabe, 1979).

In summary, the sequence of changes throughout shell repair in *Tegula* is as follows. The initial crystal deposition occurs in association with an organic matrix and appears as small, spindle-shaped crystals formed by the aggregation of needle-like subunits. The spindles are frequently aggregated into stellate clusters that coalesce to form a sheet of mineralized tissue. After about two months, dumbbell-shaped crystal aggregates and spherulites are apparent on the surface of the regenerated shell. A normal shell structure is present after at least four months of regeneration. Crystal deposition also occurs on the normal shell surrounding the window.

A salient question arises from this study. What degree of control does an animal have over the microarchitecture of regenerated shell if at any stage the ultrastructural appearance is similar to that described for inorganically precipitated mineral?

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